Cell, Vol. 87, 929–939, November 29, 1996, Copyright ©1996 by Cell Press

c-Jun Can Recruit JNK to Phosphorylate Dimerization Partners via Specific Docking Interactions

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Summary

Structurally related serine/threonine kinases recognize similar phosphoacceptor peptides in vitro yet in vivo, they phosphorylate distinct substrates. To understand the basis for this specificity, we studied the interaction between the Jun kinases (JNKs) and Jun proteins. JNKs phosphorylate c-Jun very efficiently, JunD less efficiently, but they do not phosphorylate JunB. Effective JNK substrates require a separate docking site and specificity-conferring residues flanking the phosphoacceptor. The docking site increases the efficiency and specificity of the phosphorylation reaction. JunB has a functional JNK docking site but lacks specificity-conferring residues. Insertion of such residues brings JunB under JNK control. JunD, by contrast, lacks a JNK docking site, but its phosphoacceptor peptide is identical to that of c-Jun. Substrates such as JunD can be phosphorylated by JNK through heterodimerization with docking competent partners. Therefore, heterodimerization can affect the recognition of transcription factors by signal-regulated protein kinases.

Introduction

Protein phosphorylation is a major mechanism for controlling gene expression in response to extracellular stimuli (Hill and Treisman, 1995; Karin and Hunter, 1995) and cell cycle progression (Nigg, 1995; Morgan, 1995). In both cases, regulation is achieved through phosphorylation of specific substrates by either signal-activated or cell–cycle dependent protein kinases. It is thought that different extracellular stimuli elicit distinct transcriptional responses by activating unique protein kinases that specifically phosphorylate sequence-specific transcription factors (Hill and Treisman, 1995; Karin and Hunter, 1995). Likewise, cell cycle–regulated events are executed via stage-specific phosphorylation of distinct substrates (Nigg, 1995). The existence of multiple structurally related signal activated or cell cycle–dependent protein kinases creates the difficult conceptual problem of understanding how biochemical and biological specificity is achieved. Despite large advances in understanding protein kinase structure (Taylor and Radzio-Andzelm, 1994) and mechanism of activation and catalysis (Taylor et al., 1995; Cobb and Goldsmith, 1995), the basis for specific substrate phosphorylation is generally not well understood. A notable exception among transcription factor substrates comprises the STATs (for signal transducers and activators of transcription), which are phosphorylated and activated by the janus kinases (JAKs) (Ihle et al., 1994; Darnell et al., 1994). The specificity of STAT activation does not lie in JAK–STAT interactions, but in the STAT Src homology 2 (SH2) domains, which interact with specific phosphotyrosines on the cytoplasmic domains of activated cell surface receptors (Heim et al., 1995; Stahl et al., 1995). The activated receptor recruits JAKs to other phosphotyrosine sites, thereby facilitating a productive encounter between receptor-specific STATs and whichever JAKs it binds.

Mitogen-activated protein kinases (MAPKs) also play a major role in regulation of transcription factor activity in response to extracellular stimuli (Karin and Hunter, 1995; Hill and Treisman, 1995). MAPKs have been implicated in phosphorylation and activation of a diverse array of transcription factors in mammals and yeasts (Hill and Treisman, 1995; Herskowitz, 1995). In cases such as c-Jun in mammals (Karin, 1995) and STE12 in budding yeast (Herskowitz, 1995), the transcription factor target is specifically phosphorylated by a distinct MAPK or a subgroup of closely related MAPKs. This results in a precise transcriptional response of narrow specificity. The basis for this high level of specificity is not well understood. In other cases, however, a single transcription factor, ternary complex factor (TCF)/Elk-1 for example, is phosphorylated by several MAPKs that respond to different stimuli (Cavigelli et al., 1995; Whitmarsh et al., 1995). This results in a transcriptional response of broad specificity. Comparison of different MAPK phosphoacceptor sites and the sequences that surround them, as well as the use of oriented peptide libraries, revealed little about the basis for substrate-specific recognition, except for a requirement for a proline at the P + 1 position (Davis, 1993; L. Cantley, personal communication). Interestingly, proline at the P + 1 position is also required by the cyclin-dependent kinases (CDKs; Nigg, 1993). The optimal substrate peptides for two distinct CDKs (CDK2–Cyclin B and CDK2–Cyclin A) are also very similar (Songyang et al., 1994). It is unlikely that small differences in site preference explain the distinct biological activities of these CDKs. A more likely explanation, which requires further testing, is that the cyclin subunits recruit the CDKs to specific sets of substrates (Peeper et al., 1993).

We have been studying the regulation of c-Jun, a subunit of the dimeric transcription factor AP-1, as a paradigm for the control of transcription factor activity by phosphorylation (Karin, 1995). The *jun* gene family includes *junB* and *junD*, whose products also dimerize with Fos proteins and bind AP-1 sites. These proteins form Jun–Jun homo- and heterodimers whose stability

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is lower than that of Jun–Fos heterodimers (Angel and Karin, 1991). Despite similar sequence recognition properties (Ryseck and Bravo, 1991), the Jun proteins differ in their ability to activate AP-1 dependent promoters (Chiu et al., 1989) and cooperate with Ha-Ras in oncogenic transformation (Schutte et al., 1989; Pfarr et al., 1994). Transcriptional activation by c-Jun is strongly enhanced by its phosphorylation at serines 63 and 73. Enhanced phosphorylation of S63/73 is also the basis for oncogenic cooperation between c-Jun and Ha-Ras (Smeal et al., 1991). The use of dominant-negative Jun proteins (Lloyd et al., 1991) and c-*jun*-null fibroblasts indicates that functional c-Jun is essential for Ha-Ras (Johnson et al., 1996) and v-Src (E. Wagner, personal communication) transformation. The equivalents of S63/73 are conserved among all vertebrate Jun proteins, including JunB and JunD, raising the question of why these proteins cannot cooperate with Ha-Ras or v-Src.

c-Jun is phosphorylated at S63/73 by the Jun kinases (JNKs), which belong to the MAPK family. No other MAPKs were found to be involved in c-Jun N-terminal phosphorylation in mammalian cells (Hibi et al., 1993; Dérijard et al., 1994). Efficient phosphorylation of c-Jun by JNK requires a docking site located between amino acids 30 and 79 of c-Jun (Hibi et al., 1993). This region of c-Jun interacts most efficiently with a segment of JNK2 that is located next to its catalytic pocket (Kallunki et al., 1994). We proposed that the docking site of c-Jun is used to attract the enzyme to its substrate, increasing its effective local concentration and thereby facilitating c-Jun phosphorylation (Karin, 1995). The use of docking interactions to facilitate enzymatic reactions is a common motif in signal transduction. For example, Raf activation is facilitated by its recruitment to the plasma membrane, presumably placing it in the vicinity of another protein kinase (Leevers et al., 1994). The recruitment of the exchange factor SOS to the plasma membrane places it close to its substrate Ras and is essential for Ras activation (Aronheim et al., 1994). Likewise, the cytoplasmic domain of activated cytokine receptors places the JAKs next to the STATs (Stahl et al., 1995; Heim et al., 1995).

We examined the interaction of JNK with c-Jun in further detail and explored its ability to phosphorylate and activate other Jun proteins. We find that the specific phosphorylation of c-Jun at S63/73 requires a bipartite interaction with JNK, mediated by the JNK docking site, which can be separated from the phosphoacceptor region, and by specific residues that flank the phosphoacceptor sites. In addition to increasing the efficiency of phosphorylation, the docking site is required for specific selection of phosphoacceptor sites. While JunB has an effective JNK docking site, it cannot be phosphorylated by the JNKs, owing to the absence of specificity conferring residues surrounding its phosphoacceptors. JunD, on the other hand, lacks an effective docking site, but its phosphoacceptor region is essentially identical to that of c-Jun. As a result, JunD is only weakly phosphorylated following JNK activation. We provide evidence that c-Jun and JunB can recruit JNK to phosphorylate other proteins that lack a JNK docking site, such as JunD.

Results

JunB Does Not Respond to JNK Activation

In the absence of c-Fos, JunB is an inefficient activator of the AP-1-dependent collagenase promoter, compared with c-Jun (Chiu et al., 1989). In part, this is due to inefficient dimerization and a decreased affinity for the consensus AP-1 site, caused by differences between the DNA-binding and -dimerization domains of JunB and c-Jun (Deng and Karin, 1993). We compared the effects of ultraviolet (UV) irradiation, which stimulates endogenous JNK and c-Jun phosphorylation (Devary et al., 1992; Hibi et al., 1993), on the transcriptional activities of c-Jun, JunB, and a JunB/c-Jun chimera, BC2, which contains the JunB activation and the c-Jun DNA-binding domain (Deng and Karin, 1993). While the transcriptional activity of c-Jun was readily stimulated, the activities of JunB or the BC2 were not altered by UV irradiation (Figure 1A). The failure of JunB and BC2 to respond to UV suggested that this stimulus may not affect the phosphorylation of the JunB activation domain. Digests of phosphorylated BC2 isolated from transiently transfected and UV irradiated F9 cells contained only two major phosphopeptides that migrate similarly to phosphopeptides b and c of c-Jun (Figure 1B). These phosphopeptides reflect constitutive phosphorylation of sites that are located next to the DNA-binding domain of c-Jun, which are present in the BC2, while digests of ^{32}P-labeled c-Jun isolated from UV-irradiated F9 cells also contained two other major phosphopeptides, X and Y (corresponding to serines 73 and 63).

JunB Lacks Residues Required for Phosphorylation by JNK

To identify why JNK activation does not result in N-terminal JunB phosphorylation, we compared the sequences surrounding the JNK phosphoacceptors of c-Jun S63/73 with the corresponding region of mouse and human JunB (Figure 2A). While both serines are conserved in JunB, several of the residues that surround them are not. As the JNKs are MAPKs, they are expected to be proline-directed (Davis, 1993). The most striking difference between c-Jun and JunB is the absence of prolines in JunB after the equivalents of S63 and S73. To determine the importance of these changes we inserted a P codon after the S codon equivalent to S63 and replaced the T codon that follows the S73 equivalent of JunB with a P codon. To maintain the same number of residues in JunB, we deleted a T codon located at the P + 3 position of the S63 equivalent. The resulting mutant, JunB*, has sequences identical to those of c-Jun not only at the P + 1 positions but also at many other positions. These changes were sufficient to confer upon the JunB activation domain (fused to the c-Jun DNA-binding domain; B*C2) the ability to respond to UV irradiation (Figure 2B). Tryptic phosphopeptide mapping of B*C2 expressed in F9 cells indicated that its N-terminal phosphorylation was stimulated upon UV irradiation, as revealed by the appearance of two phosphopeptides, Xb and Yb, that are not present in BC2 (Figure 2C). While the mobility of phosphopeptide Xb is identical to that

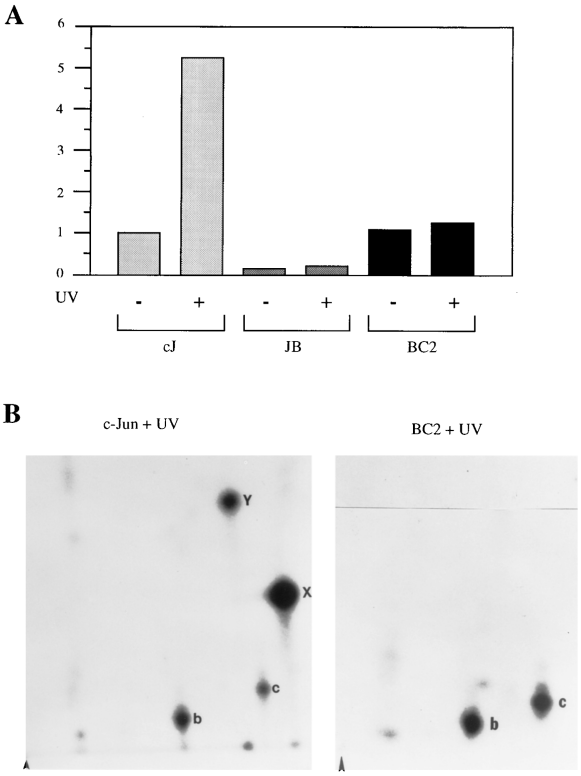

Figure 1. JunB Is Not Responsive to UV Irradiation

(A) F9 cells were cotransfected with −73Col-Luc reporter and expression vectors for c-Jun (cJ), JunB (JB), or a JunB/c-Jun chimera (BC2) containing the JunB activation domain and c-Jun DNA-binding and dimerization domain. After 10 hr the cells were UV-C irradiated (40 J/m^{-2}) and collected 8–10 hr later to determine luficerase activity. The results are presented as fold activation relative to basal c-Jun activity in nonirradiated cells.

(B) c-Jun and BC2 expression vectors were transfected into F9 cells that were labeled with [^{32}P]orthophosphate 12 hr later. After 4 hr, the cells were UV-C irradiated and collected 30 min later. After immunoprecipitation, the proteins were digested with trypsin and subjected to two-dimensional phosphopeptide mapping. The maps were visualized by autoradiography. Phosphopeptide Y contains phospho-S63, whereas phosphopeptide X contains phospho-S73. Phosphopeptides b and c contain the C-terminal phosphorylation sites. When c-Jun is isolated from nonirradiated cells, the intensity of the X and Y spots is much weaker (Devary et al., 1992). The maps of BC2 isolated from nonirradiated cells are virtually identical to the map shown above.

of phosphopeptide of c-Jun, the mobility of phosphopeptide Yb was different from that of phosphopeptide Y of c-Jun. This difference is consistent with the difference in the sequence of the two tryptic peptides. To confirm that Xb and Yb reflect phosphorylation of B*C2 at the equivalents of S73 and S63, respectively, we prepared the single mutants JB20 and JB21 (see Figure 3) and compared their phosphorylation patterns after transient expression in F9 cells (Figure 2D).

To examine the phosphorylation of the different proteins by purified JNK in vitro, we expressed the N-terminal domains of JunB and JunB* as GST fusion proteins. While GST–JB(1–153) was not phosphorylated by JNK, GST–JB*(1–154) was (Figure 2E). Thus, the prolines at the P + 1 position are essential for recognition by JNK. Since efficient phosphorylation of c-Jun appears to require a docking site to which JNK can bind (Hibi et al., 1993), we examined whether the failure of JunB to be phosphorylated is due to a defect in kinase binding. The different GST fusion proteins immobilized on beads were incubated with extracts of UV-irradiated HeLa cells, and after extensive washing, the bound proteins were eluted and examined for their ability to phosphorylate a c-Jun substrate. Similar amounts of JNK were bound by GST–cJun(1–223) and the two GST–JB fusion proteins (Figure 2F). We also incubated cell-free translated and ^{35}S-labeled c-Jun, JunB, and JunD with GST–JNK2 beads and measured the amount of bound protein (Figure 2G). Binding of JunB to JNK2 was 80% as efficient as the binding of c-Jun, while binding of JunD was very inefficient (only 4% of the c-Jun level).

Residues Flanking the Phosphoacceptor Site Determine the Efficiency of Jun Phosphorylation without Affecting JNK Binding

To determine which residues in addition to the prolines at P + 1 positions govern the specificity and efficiency of Jun phosphorylation by JNK, a series of mutants affecting other residues that follow the S73 equivalent of JunB were prepared (Figure 3A). The different mutants were expressed by transient transfection in F9 cells, and their phosphorylation pattern was determined by tryptic phosphopeptide mapping after isolation from ^{32}P-labeled and UV-irradiated cells. The efficiency of N-terminal phosphorylation was determined by comparing the relative yields of the Xb phosphopeptide, which contains the S73 equivalent, with that of the X phosphopeptide of c-Jun. The results are summarized in Figure 3A; several of the representative phosphopeptide maps were already shown in Figure 2D, and more are shown in Figure 3B. While neither of the two N-terminal phosphoacceptor sites of JunB was phosphorylated in UV-irradiated HeLa cells, the Yb site (S63 equivalent) of mutant JB20 is phosphorylated as efficiently as S63 in c-Jun. Likewise, the Xb site of mutant JB21 was phosphorylated as efficiently as S73 of c-Jun. This is expected because the Xb site in JB21 is flanked by the same immediate sequence as in c-Jun.

Both of the c-Jun phosphoacceptor sites contain an acidic residue at P + 2. However, substitution of the glutamate at P + 2 of JB21 with either an aspartate (mutant JB22a) or an alanine (mutant JB22b) did not make much of a difference. S73 is also followed by another negatively charged residue at P + 4 and a positively charged residue at P + 5, while the secondary JNK phosphoacceptor site, S63, contains noncharged residues at these positions. Replacement of the arginine at P + 5 of JB21 with a glutamate (mutant JB24) completely abolished phosphorylation of Xb (Figure 3A). When this mutation was examined within the context of JunB*, which has both the Xb and Yb sites, it abolished Xb phosphorylation without affecting phosphorylation of Yb (Figure 3B). On the other hand, replacing the leucine at P + 3 with an arginine (mutant JB25) further potentiated phosphorylation at Xb. Phosphorylation at Xb was also abolished by replacing the arginine at

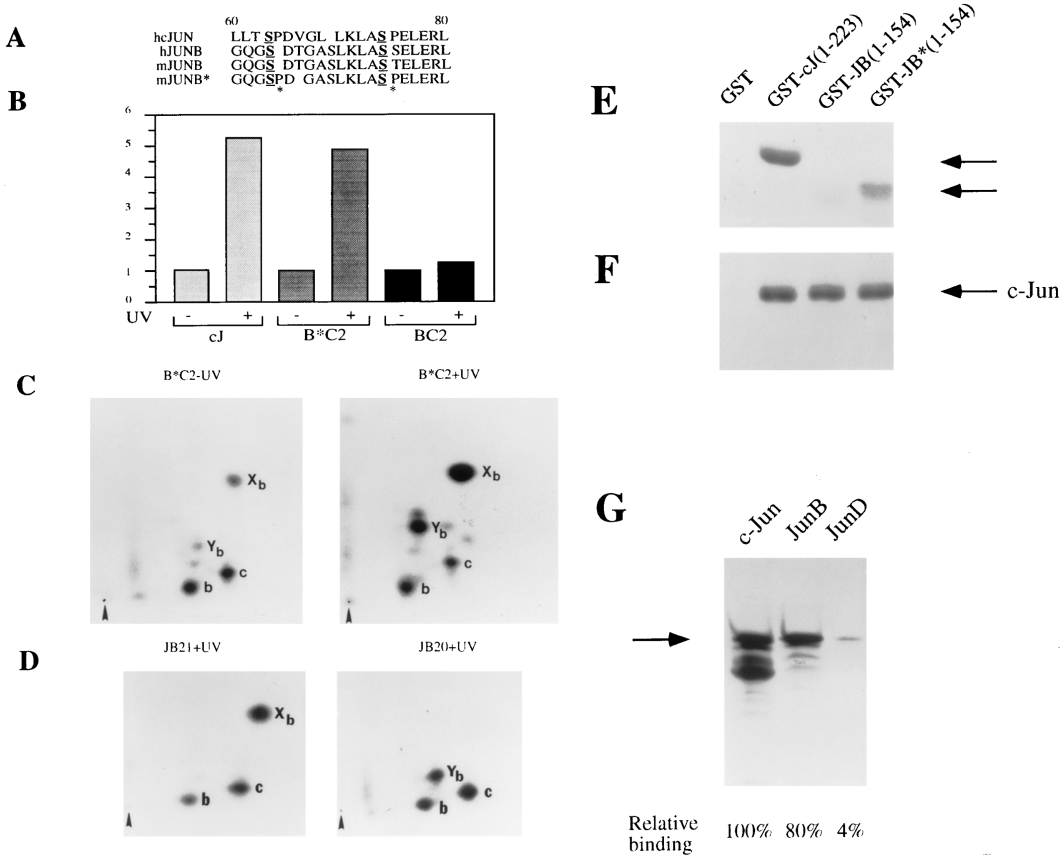

Figure 2. Insertion of Prolines at the P + 1 Positions of JunB Converts It to a UV-Responsive Transcriptional Activator

(A) Sequence comparison of the phosphoacceptor region of human c-Jun, human JunB, and mouse JunB. The numbers refer to the c-Jun sequence. Also shown is the sequence of the proline insertion and substitution mutant mJunB*.

(B) Transactivation of the −73Col-Luc reporter by c-Jun, the BC2 chimera, and the proline insertion mutant B*C2. Transactivation was determined as described in the legend to Figure 1A.

(C) Phosphopeptide maps of B*C2 isolated from ^{32}P-labeled F9 cells that were either exposed or not exposed to UV radiation. The experiment was done as described in the legend to Figure 1B. Xb contains the JunB equivalent of S73 and Yb the equivalent of S63.

(D) Phosphopeptide maps of the single mutants JB20 and JB21 isolated from ^{32}P-labeled and UV-irradiated F9 cells. JB20 is the proline insertion mutant, while JB21 is the proline substitution mutant (T to P). See (A) for sequence.

(E) Phosphorylation of GST–Jun proteins by JNK in vitro. GST or GST fusion proteins containing the N-terminal activation domains of c-Jun, JunB, or JunB* were incubated with JNK isolated from UV irradiated HeLa cells for 30 min in the presence of [γ-^{32}P]ATP. Phosphorylated proteins were separated by SDS–PAGE and visualized by autoradiography.

(F) The different GST–Jun proteins bind JNK. GST–Jun fusion proteins were loaded onto GSH–agarose beads and incubated with extracts of UV-irradiated HeLa cells. After extensive washing, bound proteins were eluted and tested for their ability to phosphorylate recombinant c-Jun.

(G) Binding of c-Jun, JunB, or JunD to JNK2. GST–JNK2-coated GSH–agarose beads were incubated with equal amounts of ^{35}S-labeled cell-free translated Juns. After extensive washing, bound proteins were eluted and separated by SDS–PAGE, and their relative levels were quantitated by use of a phosphoimager.

P + 5 with a leucine and the isoleucine at P + 7 with an arginine (mutant JB26). These results indicate that the arginine at P + 5 has an important effect on the efficiency of the JNK-catalyzed phosphotransfer reaction. Moving this residue to the P + 3 position enhanced phosphorylation, while moving it to P + 7 abolished phosphorylation. To rule out a possible contribution of differential phosphatase susceptibility, we examined the phosphorylation of the different JunB mutants expressed as GST fusion proteins by purified JNK in vitro. By and large, the results were similar to those of the in vivo experiments (data not shown).

We also compared the ability of the various JunB mutants to bind JNK2. The different mutants were synthesized by cell-free translation and incubated with GST–JNK2 beads. Despite the large differences in the efficiency of their N-terminal phosphorylation, there was little variation in binding of the JunB derivatives to JNK2 (Figure 3C). Thus, the sequences that surround the phosphoacceptor site, although an important determinant of the efficiency of the phosphorylation reaction, do not participate in docking JNK to its substrate.

The JNK Docking Site Is Separable from the Phosphoacceptor Region and Determines Specificity

In classical enzyme–substrate interactions, the substrate binds to the catalytic pocket of the enzyme, which in the

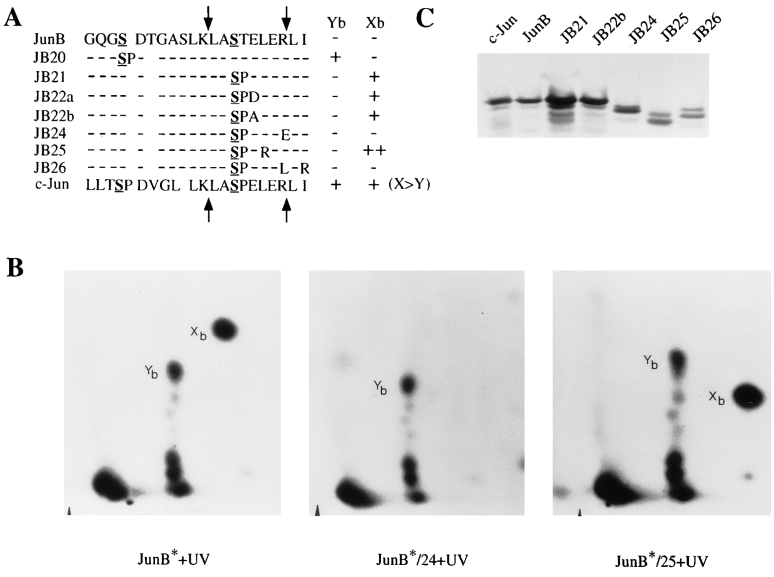

Figure 3. Residues That Flank the Phosphoacceptor Site Determine the Efficiency of JNK-Mediated Phosphorylation

(A) The different JunB phosphoacceptor region mutants and the efficiency of their phosphorylation in UV-irradiated cells. Minus, no phosphorylation; plus, phosphorylated with similar efficiency to the equivalent c-Jun site; double plus, phosphorylated more efficiently than the equivalent c-Jun site. Arrows indicate sites of tryptic cleavage. The extent of phosphorylation of the different mutants was determined by transient expression and labeling with [^{32}P]orthophosphate in F9 cells followed by UV irradiation. After 30 min, the JunB derivatives were isolated by immunoprecipitation and subjected to phosphopeptide mapping. Incorporation of ^{32}P into the N-terminal sites was compared with the amount of radioactivity in the C-terminal sites and to the labeling of the equivalent c-Jun sites in UV-irradiated F9 cells. The different mutants were expressed either in the JunB backbone or in the context of the BC2 chimera with identical results.

(B) Representative phosphopeptide maps. JunB* contains both the JB20 and the JB21 mutations. Note that owing to different C-termini, the C-terminal phosphopeptides of JunB differ in their mobilities from the b and c phosphopeptides of c-Jun.

(C) Binding of the different JunB derivatives to JNK2. Equal amounts of ^{35}S-radiolabeled cell-free translated proteins were incubated with GST–JNK2 beads. After extensive washing, the bound proteins were eluted, separated by SDS–PAGE, and visualized by autoradiography.

case of protein kinases interacts with the phosphoacceptor site and specificity-determining residues that flank it (Taylor et al., 1995). The results described above indicated that the phosphoacceptor region is not involved in docking JNK to c-Jun. Previously, we found that the first 79 amino acids of c-Jun contain both the JNK docking site and the phosphoacceptor region (Hibi et al., 1993). Further deletion analysis showed that the docking site is likely to reside between residues 30 and 60 (data not shown). Comparison of the c-Jun sequence in this region to the equivalent regions of JunB and JunD reveals that only five residues are invariant, and that JunB is much more similar in this region to c-Jun than to JunD (Figure 4A). To confirm that this region contains the docking site, we replaced four of the invariant residues in c-Jun with alanines. The resulting mutant, c-Jun(A40/42/49/50), was defective in binding to JNK2 (Figure 4B). While this mutant was expressed as efficiently as wild-type (wt) c-Jun in Jurkat cells (Figure 4C), its level of N-terminal phosphorylation was much lower than that of c-Jun and was not enhanced following UV irradiation (Figure 4D). Its inability to respond to UV is also apparent from ^{35}S labeling: while the electrophoretic mobility of wt c-Jun is retarded following UV irradiation, the electrophoretic mobility of the mutant is not affected (Figure 4C).

When we examined the JNK phosphorylation pattern of some docking site mutants, we found that most of the phosphorylation occurred on sites other than S63 (phosphopeptide Y) or S73 (phosphopeptide X). As shown in Figure 4E, the major JNK phosphorylation sites of such mutants included the C-terminal MAPK and casein kinase II sites (phosphopeptides b and c; Lin et al., 1992), S91/93 (phosphopeptides T1 and T2; Hibi et al., 1993). By contrast, wt c-Jun was phosphorylated almost exclusively at S63 and S73. Assuming that these mutants are properly folded, these results indicate that in addition to affecting the efficiency of substrate phosphorylation, the docking site is also responsible for directing the kinase to only a small subset of potential phosphoacceptors.

To establish the independence of the JNK docking site from the phosphoacceptor region, we increased the distance between them by inserting a heterologous 55-mer peptide segment (amino acids 3–57 of CREB [for cAMP response element–binding protein]) between amino acids 57 and 58 of c-Jun (Figure 5A). Phosphorylation of this mutant c-J(CREB 3–57) was stimulated by UV irradiation as efficiently as that of wt c-Jun (Figure 5B). Phosphopeptide mapping confirmed that UV induced phosphorylation was restricted to the N-terminal sites of the insertion mutant (data not shown).

Efficient N-Terminal c-Jun Phosphorylation Requires Dimerization

c-Jun exists in vivo either as a homodimer or a heterodimer with other partners (Angel and Karin, 1991). We examined the ability of JNK to phosphorylate a mutant of c-Jun, M15, that is defective in homodimerization but capable of heterodimerizing with c-Fos (Smeal et al., 1989). The level of M15 phosphorylation either by purified JNK2 or in UV-irradiated HeLa cells was considerably lower than the level of c-Jun phosphorylation (Figure 6A). Coexpression of M15 with a chimeric c-Jun protein that contains the c-Fos leucine zipper but lacks the JNK docking site, cJ(Δ56)/cFLZ, enhanced its phosphorylation (Figure 6B). The cJ(Δ56)/cFLZ by itself is very poorly phosphorylated following JNK activation (Hibi et al., 1993).

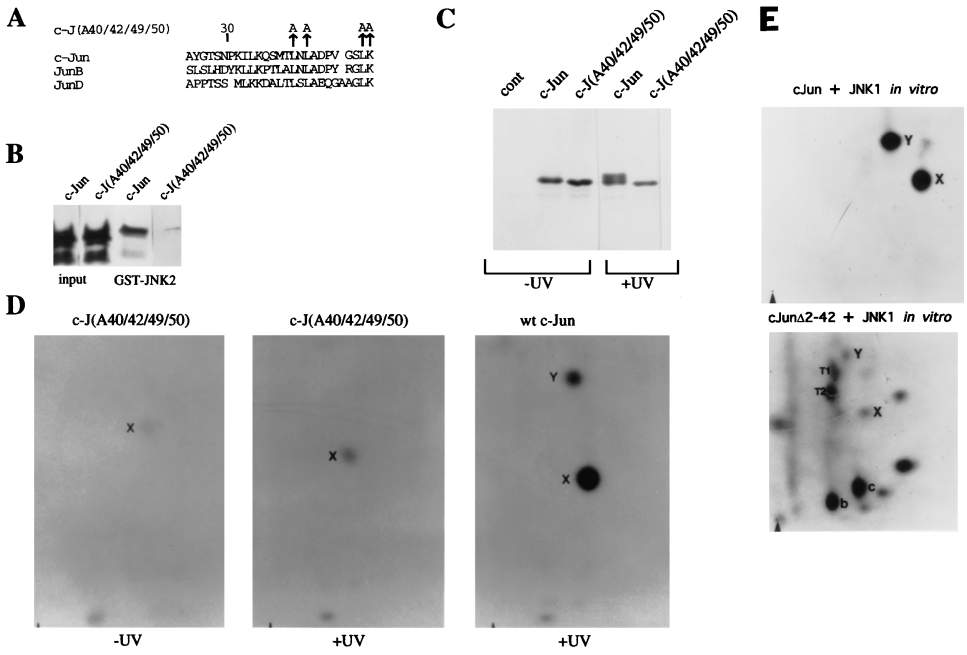

Figure 4. The N-Terminal JNK Docking Site Dictates Both the Efficiency and the Specificity of c-Jun Phosphorylation

(A) Sequence comparison of the regions involved in JNK docking in c-Jun, JunB, and JunD. Also shown are the c-Jun residues that were substituted with alanines.

(B) c-Jun(40/42/49/50) is defective in JNK binding. Equal amounts of ^{35}S-labeled cell-free translated proteins were incubated with GST–JNK2 beads. After extensive washing, the bound proteins were eluted, separated by SDS–PAGE, and visualized by autoradiography.

(C) Expression of HA-tagged wt c-Jun and c-Jun(A40/42/49/50) in Jurkat cells. Expression vectors encoding these proteins were transfected into Jurkat cells, which after 48 hr were labeled with [^{35}S]methionine for 3 hr. The cells were UV irradiated 30 min prior to immunoprecipitation of the Jun proteins with HA monoclonal antibody. The precipitates were analyzed by SDS–PAGE and autoradiography.

(D) Phosphorylation of c-Jun and c-Jun(A40/42/49/50) in Jurkat cells. The proteins were expressed and immunopurified as described above, except that the cells were labeled with [^{32}P]orthophosphate. The immunopurified proteins were subjected to peptide mapping.

(E) The docking site determines the choice of phosphoacceptor sites. Recombinant c-Jun or c-JunΔ2-42, which lacks the JNK docking site, were purified from E. coli and phosphorylated in vitro with purified JNK1. Samples containing similar amounts of ^{32}P were trypsin digested and subjected to phosphopeptide mapping. Phosphopeptides containing previously identified phosphoacceptor sites are marked. X and Y, S73 and S63, respectively; T1 and T2, T91 and T93; b and c, T239 and S243.

c-Jun Can Recruit JNK to Phosphorylate Other Substrates

JunD binds poorly to JNK in vitro (see Figure 2G). However, its phosphoacceptor region is very similar to that of c-Jun. On the basis of its relative affinity to JNK, JunD should not be phosphorylated more efficiently than c-Jun(A40/42/49/50), which exhibits the same level of JNK2 binding. However, the stimulation of JunD N-terminal phosphorylation by UV irradiation, while lower than that of c-Jun, was more substantial than that of c-Jun(A40/42/49/50) (compare Figure 7C with Figure 4D). We considered the possibility that instead of direct JNK docking, the N-terminal phosphorylation of JunD is mediated through dimerization with another protein capable of recruiting JNK, such as c-Jun or JunB. Because dimerization, per se, is required for efficient phosphorylation of c-Jun by JNK (see Figure 6), a dimerization defective mutant of JunD should not be phosphorylated at all. To examine this, we constructed the JunD(cFLZ) chimera, in which the JunD leucine zipper is replaced with the equivalent region of c-Fos (Figure 7A). As expected, JunD(cFLZ) associated with c-Jun in vitro as efficiently as c-Fos and much more efficiently than JunD (Figure 7B). When the phosphorylation pattern of JunD(cFLZ) was compared with that of JunD in Jurkat cells, cotransfected with a c-Jun vector, we found that the mutant was much more responsive to UV light. While UV irradiation enhanced the N-terminal phosphorylation of JunD(cFLZ) by 15-fold, the N-terminal phosphorylation of wt JunD was enhanced only by 3-fold (Figure 7C). The presence of the Fos leucine zipper did not enhance the binding of JunD(cFLZ) to JNK2, but unlike wt JunD, a much higher amount of this mutant associated with JNK2 in the presence of c-Jun (Figure 7D). In addition, heterodimerization with c-Jun strongly enhanced the phosphorylation of JunD(cFLZ) by JNK2 in vitro, while having only a small effect on the phosphorylation of wt JunD (Figure 7E). These results strongly support the notion that JunD is phosphorylated by JNK not through a direct docking interaction but via heterodimerization with another protein to which JNK can dock.

We also examined whether coexpression of the docking-defective mutant of c-Jun, c-Jun(A40/42/49/50), with another protein to which JNK can bind will enhance its N-terminal phosphorylation. Indeed, coexpression of c-Jun(A40/42/49/50) with JunB, which can bind JNK but cannot be phosphorylated by it, reconstituted the enhancement of N-terminal phosphorylation by UV irradiation (compare Figure 7F with Figure 4D, which shows

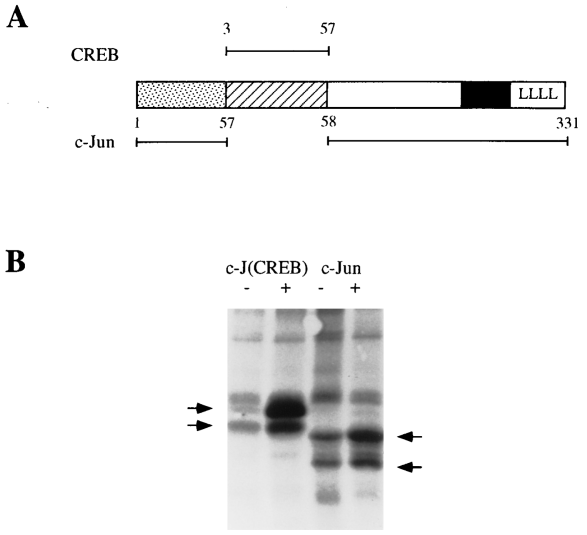

Figure 5. The JNK Docking Site Can Be Separated from the Phosphoacceptor Region

(A) A diagram illustrating the c-J(CREB3–57) insertion mutant.

(B) Phosphorylation of c-J(CREB3–57) is stimulated by UV radiation. Expression vectors encoding c-J(CREB3–57) or wt c-Jun were transfected into F9 cells. After 12 hr, the cells were labeled with ^{32}P for 4 hr and either exposed (plus) or not (minus) to UV radiation. After 30 min, the Jun proteins were immunoprecipitated, separated by SDS–PAGE, and visualized by autoradiography. The arrows indicate the intact c-J(CREB 3–57) or c-Jun proteins and a proteolytic product.

the defective response of c-Jun(A40/42/49/50) to UV irradiation).

Discussion

The Jun proteins are sequence specific transcriptional regulators (Angel and Karin, 1991). Despite almost identical DNA-binding and dimerization domains and well-conserved activation domains, these proteins respond differently to extracellular stimuli. Of the three mammalian Juns, only c-Jun is highly responsive to UV irradiation (Devary et al., 1992) or activated Ras (Smeal et al., 1991), which stimulate its phosphorylation and transcriptional activity. c-Jun is absolutely essential for proliferation of primary fibroblasts and their transformation by either Ha-Ras or v-Src (Johnson et al., 1996; E. Wagner, personal communication). These oncogenic and environmental stimuli activate the JNK subgroup of MAPKs, responsible for stimulating the transcriptional activity of c-Jun. The response of c-Jun to JNK is highly specific, as the positive regulatory sites in its activation domain are not phosphorylated by other currently known signal-responsive protein kinases (Hibi et al., 1993; Minden et al., 1994). In this study, we investigated the molecular basis for this narrow specificity and explored why the activities of other Jun proteins are not regulated by the JNKs. The results described above shed light not only on the mechanisms by which the JNKs discriminate between the different Juns but also on the general problem of substrate recognition by serine/threonine kinases.

All protein kinases interact via their catalytic pocket with the phosphoaccepting hydroxyamino acid as well as with several specificity-conferring residues that flank the phosphoacceptor site (Songyang et al., 1994; Taylor et al., 1995). While the residues that flank the phosphoacceptor site provide a certain degree of specificity, this is unlikely to be sufficient for specific recognition of a substrate of regulatory importance by one or a few members of a large family of closely related protein kinases, such as the MAPKs and CDKs. In fact, there is a high degree of similarity between phosphoacceptor sites recognized by MAPKs to those recognized by CDKs, even though the regulatory functions of these enzymes are entirely different. An even greater redundancy is found when recognition sequences for different CDKs (Songyang et al., 1994) or MAPKs (L. Cantley, personal communication) are compared with those recognized by other members of their groups. In addition to lack of sufficient specificity, it has generally been observed that even optimized peptide substrates are often recognized less efficiently (i.e., with a higher K_m) than physiologically relevant protein substrates. Such observations suggest that physiologically relevant substrates are likely to interact with their kinases through additional sites outside the phosphopeptide region, which increase the affinity of their interactions. In the

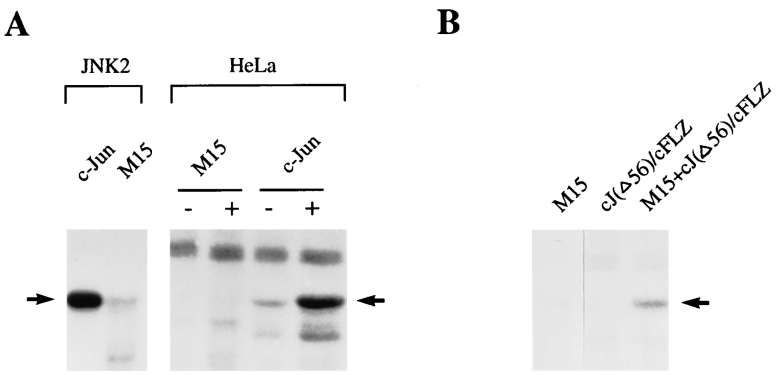

Figure 6. Efficient c-Jun Phosphorylation Requires Dimerization

(A) Recombinant c-Jun or the M15 mutant (F287/308), which is defective in homodimerization, were incubated with purified JNK2 and [γ-^{32}P]ATP. The same proteins were also transiently expressed in F9 cells that were labeled with [^{32}P]orthophosphate and either exposed (plus) or not (minus) to UV irradiation prior to immunoprecipitation. The ^{32}P-labeled proteins were resolved by SDS–PAGE and visualized by autoradiography.

(B) F9 cells were transfected with expression vectors encoding M15 or cJ(Δ56)/cFLZ, which is a c-Jun lacking the JNK docking site and whose leucine zipper was replaced with that of c-Fos, or with expression vectors encoding both proteins. The transfected cells were labeled with [^{32}P]orthophosphate and UV irradiated prior to immunoprecipitation of the various proteins. The arrow indicates the migration position of M15. The shorter cJ(Δ56)/cFLZ was not labeled.

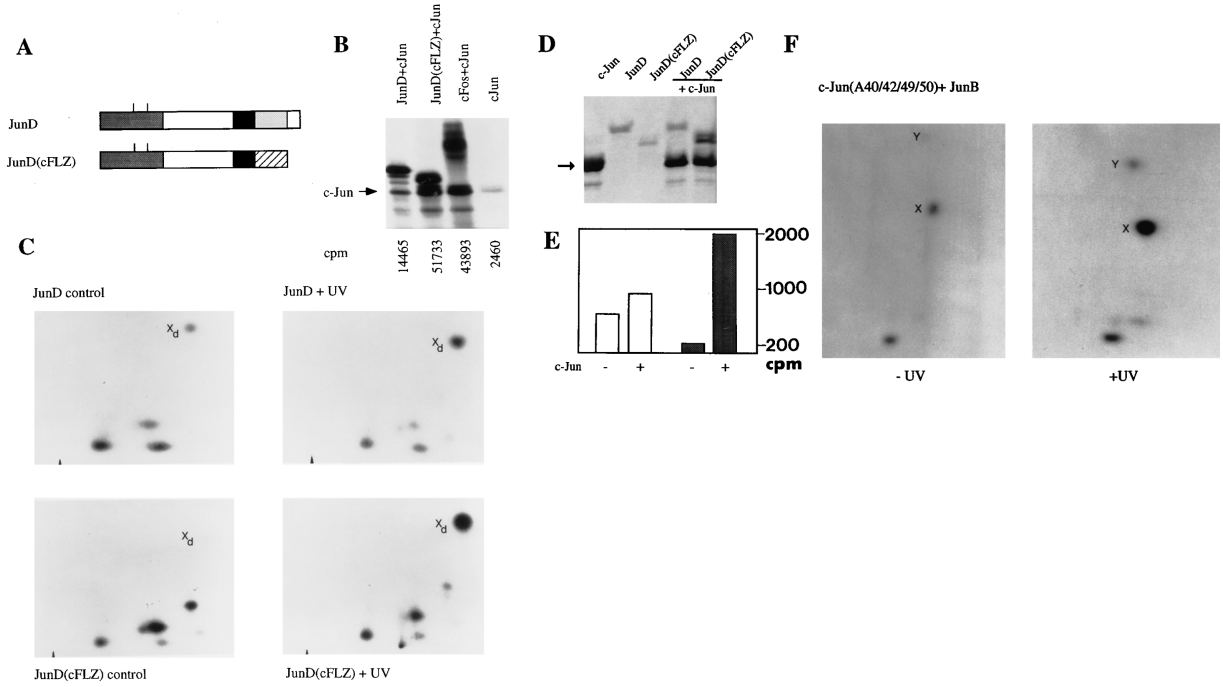

Figure 7. c-Jun and JunB Recruit JNK to Phosphorylate Other Substrates

(A) Schemes of the JunD and JunD(cFLZ) proteins. In JunD(cFLZ), the JunD leucine zipper is replaced by that of c-Fos. Activation domain, dark gray box; basic region, closed box; JunD leucine zipper, light gray box; c-Fos leucine zipper, hatched box.

(B) Dimerization of JunD, JunD(cFLZ), and c-Fos with c-Jun. Equal amounts of ^{35}S-labeled in vitro translated HA-tagged JunD, JunD(cFLZ), and c-Fos were incubated at 30°C with similar amounts of ^{35}S-labeled nontagged c-Jun for 90 min to allow dimerization. After precipitation with HA antibody and protein A–sepharose and extensive washing, the bound proteins were eluted by boiling in loading buffer and were separated by SDS–PAGE. The amounts of precipitated c-Jun were quantitated. The c-Jun band is indicated.

(C) In vivo phosphorylation of JunD and JunD(cFLZ). Jurkat cells were transfected with HA-tagged JunD or JunD(cFLZ) expression vectors and labeled with [^{32}P]orthophosphate for 3 hr, after which they were either UV irradiated or not. After 30 min, the proteins were immunoprecipitated and subjected to tryptic phosphopeptide mapping.

(D) c-Jun recruits JunD to JNK. Equal amounts of ^{35}S-labeled in vitro translated c-Jun, JunD, and JunD(cFLZ) were incubated by themselves or in the indicated combinations at 30°C for 90 min to allow dimerization. These samples were incubated with GST–JNK2 beads, extensively washed, eluted, and separated by SDS–PAGE. The arrow indicates the c-Jun band, and the different JunD proteins migrate above it.

(E) Dimerization with c-Jun enhances JunD phosphorylation in vitro. Similar amounts of in vitro translated ^{35}S-labeled HA–JunD or HA–JunD(cFLZ) were immunoprecipitated with HA antibody and eluted from the immune complex with HA peptide. The eluted proteins were incubated with or without recombinant c-Jun at 30°C for 90 min to allow dimerization, and then phosphorylated with recombinant JNK2. The proteins were separated by SDS–PAGE, and their extent of phosphorylation was determined. The figure shows the labeling of JunD (open bars) and JunD(cFLZ) (closed bars) in the absence or presence of c-Jun.

(F) JunB potentiates the phosphorylation of the docking-defective mutant c-Jun(A40/42/49/50). Jurkat cells were cotransfected with c-Jun(A40/42/49/50) and JunB expression vectors, labeled with [^{32}P]orthophosphate, and UV irradiated or not, as described in Figure 4C (experiments were done in parallel). c-Jun(A40/42/49/50) was immunoprecipitated and analyzed by phosphopeptide mapping.

case of protein tyrosine kinases, the specificity problem has been solved through a bipartite interaction with their substrates. In addition to the catalytic pocket interacting with the phosphoacceptor peptide, either an SH2 or a PID/PTB domain on the substrate specifically interacts with a phosphotyrosine on the kinase (Songyang and Cantley, 1995). The basis for specific substrate recognition by serine/threonine kinases is less clear. However, the lessons learned from the JNK–Jun interactions provide a useful framework for understanding this problem.

The results described above demonstrate that the interaction between JNK and c-Jun is also bipartite. The first step in recognition of c-Jun is mediated by docking of JNK to a specific site in c-Jun, located between amino acids 30 and 60. Mutations within this region that interfere with binding of JNK reduce the efficiency of c-Jun phosphorylation by JNK either in vitro or in vivo (Figure 4). The docking site is not a part of the phosphoacceptor region, because a heterologous 55-mer peptide can be inserted between the two without exerting a deleterious effect on the efficiency of c-Jun phosphorylation and the choice of phosphoacceptors (Figure 5). We have previously shown that JNK2 interacts with the docking site of c-Jun via a peptide loop that is not a part of its classic substrate-binding pocket (Kallunki et al., 1994). In addition to enhancing the efficiency of the phosphorylation reaction, probably by increasing the local concentration of the enzyme next to its substrate, the docking site has a strong influence on the choice of phosphoacceptor sites. In the presence of the docking site, JNK phosphorylates c-Jun only on two major sites, but in its absence, c-Jun is weakly phosphorylated on many additional and physiologically irrelevant sites, several of which become the preferred sites (Figure 4E). The

docking site of c-Jun, however, is not the sole determinant of phosphorylation efficiency. Residues that flank the phosphoacceptor sites are also very important, but have no effect on initial binding of JNK to c-Jun. In addition to a proline at P + 1, efficient phosphorylation of c-Jun by JNK either in vivo or in vitro requires an arginine at P + 5 (Figure 3). Moving this arginine closer to the phosphoacceptor site (to P + 3) enhances phosphorylation, while moving it further away (to P+7) abolishes phosphorylation. Although identification of the optimal JNK phosphoacceptor sequence requires further analysis, these results clearly illustrate that the phosphoacceptor region is an important specificity determinant and that it is not involved in JNK docking. This conclusion is further affirmed by the case of JunB, which has an efficient JNK docking site but is not phosphorylated by it because it lacks prolines following its S63/73 homologs. The docking of JNK to its substrates limits its ability to recognize potential phosphoacceptor sites to those that are located within a certain distance from the docking site. However, as discussed below, these sites can also be located on another molecule. The bipartite mechanism for c-Jun phosphorylation by JNK is summarized in Figure 8A. The first step involves the recognition of the docking site by a putative substrate recognition loop that is located between kinase subdomains IX and X outside its putative catalytic pocket (Kallunki et al., 1994). This step results in high local concentration of the enzyme next to its substrate, thus facilitating the second step, which involves recognition of the phosphoacceptor region by the catalytic pocket (Taylor et al., 1995; Taylor and Radzio-Andzelm, 1994). This interaction is probably of lower affinity than the initial docking interaction. The third step is the phosphotransfer reaction followed by dissociation of the enzyme substrate complex.

A bipartite recognition mechanism is likely to be applicable to other cases of highly specific substrate phosphorylation by serine/threonine kinases. For example, ATF2 also has a JNK docking site required for efficient phosphorylation (Gupta et al., 1995). It was shown that the specific phosphorylation of E2F-1/DP-1 by CDK–Cyclin A requires a cyclin A–binding sequence. E2F-1/DP-1 is not phosphorylated by other CDK–cyclin complexes (Krek et al., 1994), probably because its docking site is highly specific to cyclin A. More recently, CDC2 was shown to directly bind a potential substrate, ORC2 (Leatherwood et al., 1996). Another example for the importance of kinase docking is the specific phosphorylation of the β-adrenergic receptor by its kinase, βARK, which is facilitated by βARK binding to $G_{\beta\gamma}$ (Inglese et al., 1995).

Our results also provide a satisfactory explanation for the differences in the ability of the Juns to respond to extracellular stimuli. Only c-Jun is efficiently phosphorylated and stimulated by the JNK pathway, because it is the only Jun that contains both an effective docking site and a favorable phosphoacceptor region. JunB has an efficient docking site, but its phosphoacceptor region cannot be recognized by the JNKs or other MAPKs, while JunD contains a functional phosphoacceptor region, but its docking site interacts with JNKs very poorly. It is possible that JunD may be recognized by other MAPKs, whereas JunB may serve other functions, such as recruiting JNK to other targets in cells that do not express c-Jun, as well as being a target to a different signaling pathway.

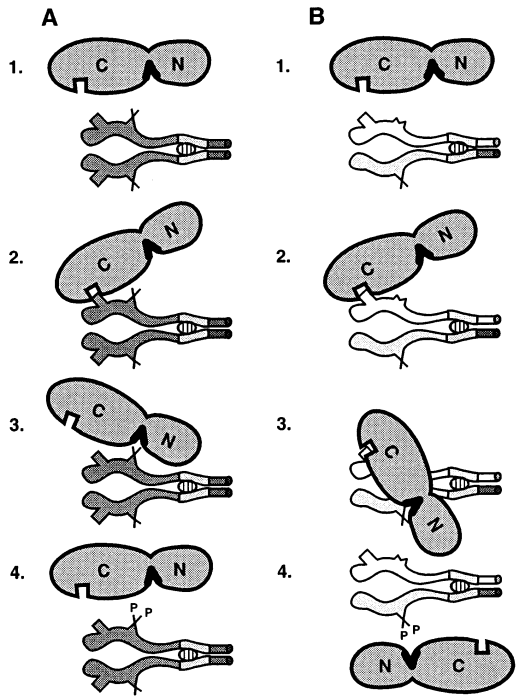

Figure 8. Models Describing Phosphorylation of Jun Proteins by JNK

(A) Phosphorylation of c-Jun. The docking site is depicted by the rectangular extrusion, while the phosphoacceptor region is indicated by the triangular extrusion and the two spikes. The catalytic pocket of JNK is located near the cleft between its N- and C-terminal lobes and is marked by the black V. The substrate recognition sequence in the C-terminal lobe is depicted by the rectangular indentation. JNK first interacts with the docking site of c-Jun. This transient interaction increases the local concentration of the substrate next to the enzyme. After JNK dissociates from the docking site, it can reinteract via its catalytic pocket with the phosphoacceptor region of c-Jun, resulting in c-Jun phosphorylation. This is followed by dissociation of the kinase.

(B) Phosphorylation of a substrate lacking a docking site, such as JunD. In this case, JNK first interacts with the docking site of the heterodimerization partner (c-Jun or JunB). After JNK dissociates from the docking site, it can interact with the phosphoacceptor site of JunD, resulting in its phosphorylation and dissociation of the kinase–substrate complex.

The most novel aspect of our results is the ability of c-Jun and JunB to recruit JNK to phosphorylate other targets via heterodimerization (Figure 8B). Despite the absence of an effective JNK docking site, JunD phosphorylation is weakly stimulated in living cells in response to JNK activation. This low level of phosphorylation could be due to heterodimerization of JunD with a partner capable of recruiting JNK, such as c-Jun or JunB. Increasing the ability of JunD to heterodimerize with other Juns, strongly increases its ability to be phosphorylated in vivo or in vitro by the JNKs (Figures 7C and 7E). The c-Fos leucine zipper allows the chimeric JunD(cFLZ) protein to be recruited to JNK more efficiently by forming a heterodimer with c-Jun (Figure 7D).

It is unlikely that the c-Fos leucine zipper affects the phosphorylation of JunD(cFLZ) by another mechanism, because it is well removed from the N-terminal phosphoacceptor sites. Also, because JNK preferentially phosphorylates c-Jun dimers (Figure 6), the c-Fos leucine zipper, incapable of homodimerization, decreases the phosphorylation of JunD (as seen in vitro, Figure 7E), unless it heterodimerizes with another protein. Further evidence for JNK recruitment through heterodimerization is provided by the experiment in which overexpression of JunB rescues the ability of a docking-defective mutant of c-Jun to be phosphorylated by JNK (Figure 7F).

These findings illustrate a new property of heterodimeric transcription factors. In addition to affecting DNA binding activity, sequence specificity, and interactions with the basal transcriptional machinery, heterodimerization also modulates responses to extracellular cues. The actual response of a given transcription factor to signaling pathways not only is dependent on intrinsic determinants, such as docking sites and phosphoacceptor sites, but is also highly influenced by its dimerization partners. This important property greatly enhances the regulatory diversity of these ultimate effectors of signal transduction cascades.

Experimental Procedures

Plasmids

The −73 Col-Luc reporter and expression vectors for c-Jun, JunB/c-Jun chimera (BC2), and JunB were described (Deng and Karin, 1993). The JunB mutants (see Figure 3A) were generated by site-directed mutagenesis of the BC2 construct or wt JunB by established procedures (Deng and Karin, 1993). c-Jun mutant M15 was described (Smeal et al., 1989). c-JunΔ2–42 was made by deleting amino acids 2–42 of c-Jun. c-J(CREB 3–57) was created by inserting a fragment of CREB encoding amino acids 3–57 between amino acids 3 and 57 of c-Jun. c-Jun (A40/42/49/50) was made by site-directed mutagenesis of wt c-Jun. Hemagglutinin (HA)-tagged c-Jun, JunB, JunD, and c-Fos were constructed by fusing the cDNAs to a 3× HA oligonucleotide in an SRα3 expression vector. cJ(Δ56)/cFLZ was constructed by deleting the first 56 amino acids of c-Jun and fusing this in-frame with the c-Fos leucine zipper. JunD(cFLZ) was generated by fusing JunD sequences from codons 1 to 289 (including the basic region) to the c-Fos leucine zipper. Further details are available upon request. The sequence of all junctions and mutated regions was confirmed by sequencing.

Transfections and In Vivo Labeling

F9 cells were maintained and transfected as described (Deng and Karin, 1993). For in vivo labeling, transfected F9 cells were placed in phosphate-free medium, 0.5% FCS 12 hr after transfection. After 30 min, [^{32}P]orthophosphate was added, and the cells were labeled for 4 hr, exposed to 40 J/m^{-2} UV-C, and collected 30 min later. Jurkat-Tag, HeLa S3, and COS7 cells were maintained and transfected as described (Kallunki et al., 1994). Jurkat-Tag cells were labeled as F9 cells.

In Vitro Translations and Expression of GST Fusion Proteins

In vitro translations were performed with the TNT SP6 Coupled Reticulocyte Lysate System (Promega). After translation, the proteins were analyzed by SDS–polyacrylamide gel electrophoresis (PAGE) and quantitated by phosphoimager. GST fusion proteins were expressed and purified as described (Hibi et al., 1993).

Phosphorylation and Binding Assays

Phosphopeptide mapping was performed as described (Lin et al., 1992). Kinase and binding assays were performed as described (Hibi et al., 1993; Kallunki et al., 1994), but instead of GST–cJun, GST–JNK2 beads were used to bind in vitro translated Jun proteins.

Acknowledgments

Correspondence should be addressed to M. K. We thank Tony Hunter and Susan Taylor for comments and discussion, Lew Cantley for sharing unpublished results, and Ebrahim Zandi for recombinant JNK2 protein. T. K. was supported by a postdoctoral fellowship from the American Heart Association (AHA), California Affiliate, and T. D. by the AHA, Florida Affiliate. Work was supported by grant CA-50528 from the National Institutes of Health.

Received August 15, 1996; revised September 27, 1996.

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